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Adjuvant Immunotherapy With Autologous Cytokine-Induced Killer Cells for Hepatocellular Carcinoma

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BACKGROUND & AIMS: No adjuvant therapy has been shown to extend the survival of patients with hepatocellular carcinoma (HCC) receiving curative treatment. We investigated whether injections of activated cytokine-induced killer (CIK) cells (CD3+/CD56+ and CD3+/CD56- T cells and CD3-/CD56+ natural killer cells) prolongs recurrence-free survival of patients after curative therapy for HCC. **METHODS:** We performed a multicenter, randomized, open-label, phase 3 trial of the efficacy and safety of adjuvant immunotherapy with activated CIK cells (created by incubation of patients' peripheral blood mononuclear cells with interleukin 2 and an antibody against CD3). The study included 230 patients with HCC treated by surgical resection, radiofrequency ablation, or percutaneous ethanol injection at university-affiliated hospitals in Korea. Patients were assigned randomly to receive immunotherapy (injection of 6.4×10^9 autologous CIK cells, 16 times during 60 weeks) or no adjuvant therapy (controls). The primary end point was recurrence-free survival; secondary end points included overall survival, cancer-specific survival, and safety. **RESULTS:** The median time of recurrence-free survival was 44.0 months in the immunotherapy group and 30.0 months in the control group (hazard ratio with immunotherapy, 0.63; 95% confidence interval [CI], 0.43–0.94; $P = .010$ by 1-sided log-rank test). Hazard ratios also were lower in the immunotherapy than in the control group for all-cause death (0.21; 95% CI, 0.06–0.75; $P = .008$) and cancer-related death (0.19; 95% CI, 0.04–0.87; $P = .02$). A significantly higher proportion of patients in the immunotherapy group than in the control group had an adverse event (62% vs 41%; $P = .002$), but the proportion of patients with serious adverse events did not differ significantly between groups (7.8% vs 3.5%; $P = .15$). **CONCLUSIONS:** In patients who underwent curative treatment for HCC, adjuvant immunotherapy with activated CIK cells increased recurrence-free and overall survival. ClinicalTrials.gov number: NCT00699816.

Keywords: Liver Cancer; Clinical Trial; IL2; NK Cell.

The implementation of surveillance programs for early detection of hepatocellular carcinoma (HCC) in high-risk populations has increased the likelihood of curative treatment.[1,2] However, the long-term prognosis still is poor even after a curative treatment because of the high frequency of recurrence in the remnant liver.[3] This high recurrence rate has led efforts to develop adjuvant therapies to reduce recurrence. However, the benefit of any form of adjuvant therapy remains unclear,[4,5] and current scientific guidelines do not recommend adjuvant therapy after curative treatment.[6–8]

Regarding adjuvant adoptive immunotherapy, cytokine-induced killer (CIK) cell–based immunotherapy has become a promising novel strategy. CIK cells are a mixture of T lymphocytes, which are ex vivo expanded with cytokines, comprising $CD3^+/CD56^+$ cells, $CD3^-/CD56^+$ natural killer (NK) cells, and $CD3^+/CD56^-$ cytotoxic T cells. Among them, $CD3^+/CD56^+$ T cells, which are rare in uncultured peripheral blood, are the main effector cells.[9] They have a high proliferation rate, potent antitumor effects with the dual-functional capability of both T cells and NK cells, and little cytotoxicity to normal cells, but with substantial specificity to tumor cells.[10,11] Earlier preclinical and clinical studies also showed a potent antitumor activity of CIK cells against various tumors.[12–14] A previous clinical trial from Japan reported that CIK cell immunotherapy increased recurrence-free survival (RFS) after surgical resection of HCC.[15] Similar results were reproduced in 2 other clinical trials.[16,17] Preceding preclinical studies showed that CIK cells killed HCC cells in vitro,[18] were localized in cancer mass in vivo,[19] and induced no major side effects after repeated transfer.[20] Encouraged by these promising results, the manufacturing techniques were refined and standardized, and an individualized autologous CIK cell–based immunotherapeutic agent (Immuncell-LC; Green Cross Cell Corp, Seoul, Korea) was developed. This CIK cell agent is

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Abbreviations used in this paper: AE, adverse event; CI, confidence interval; CIK, cytokine-induced killer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; IL2, interleukin 2; MHC, major histocompatibility complex; NK, natural killer; OS, overall survival; PEI, percutaneous ethanol injection; RFA, radiofrequency ablation; RFS, recurrence-free survival.

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manufactured by extracorporeal culture of respective patients' peripheral blood mononuclear cells with co-stimulation using interleukin 2 (IL2) and anti-CD3 antibody.

In this study, we aimed to assess the efficacy and safety of the CIK cell agent as an adjuvant therapy for HCC.

Materials and Methods

Patients

Patients who had undergone curative treatment (surgical resection, radiofrequency ablation [RFA], or percutaneous ethanol injection [PEI]) for HCC of pretreatment clinical stage I or II according to the American Joint Committee on Cancer staging system (6th edition) based on radiologic imaging studies were eligible for this study (Supplementary Table 1).[21] The diagnosis of HCC was made by pathologic examination or radiologic imaging studies.[22] Eligibility criteria also included hepatic function of Child–Pugh class A, an Eastern Cooperative Oncology Group performance status score of 0 or 1, and age between 20 and 80 years. Exclusion criteria included patients with immune deficiency or autoimmune diseases, previous or current other malignancies, and severe allergic disorder. Pregnant or breast-feeding women and women planning to get pregnant also were excluded.

Trial Design and Treatment

All participants provided written informed consent before enrollment. The study protocol was approved by the institutional review board at each participating center. All methods and procedures associated with this study were conducted in accordance with the Good Clinical Practice guidelines and accorded ethically with the principles of the Declaration of Helsinki and local laws. All authors had access to the study data and reviewed and approved the final manuscript.

This phase 3 clinical study was a multicenter, randomized, open-labeled trial. The study was conducted at 5 university-affiliated hospitals in Korea. All eligible participants were assigned randomly, in a 1:1 ratio, to receive adjuvant adoptive immune therapy using a CIK cell agent (the immunotherapy group) or no adjuvant treatment (the control group). Random assignment was performed through a central telephone system using computer-generated, permuted blocks with a block size of 4 or 6 and stratified according to study center.

During the pretreatment period, peripheral blood (120 mL) for manufacturing the individualized CIK cell agent was collected from the respective patients who were randomized to the immunotherapy group at least 4 weeks before starting treatment. The CIK cell agent was prepared at a central manufacturing facility. Mononuclear cells were separated and cultured for 12–21 days with IL2 and immobilized monoclonal antibody to CD3 at 37°C according to a modified protocol of the original method (Supplementary Figure 1).[10,23] Patients in the immunotherapy group received 200 mL of the CIK cell agent intravenously over 60 minutes without any premedication and then were observed for at least 30 minutes. They were scheduled to receive the CIK cell agent 16 times (4 treatments at a frequency of once per week, followed by 4 treatments every 2 weeks, then 4 treatments every 4 weeks, and finally 4 treatments every 8 weeks). Treatment could be delayed for a maximum of 2 weeks if the CIK cell agent was not manufactured appropriately (Supplementary Table 2). Cytokines such as interferon, chemotherapy agents, other immunotherapy agents, hormonal therapy, and stem cell therapy were contraindicated during the study.

End Points and Assessments

The primary end point was RFS. RFS was measured from the date of randomization to the first recurrence or to death from any cause. The secondary end points included overall and cancer-specific survivals and safety. Overall survival (OS) was measured from the date of randomization until death from any cause, and cancer-specific survival was measured from the date of randomization until death resulting from HCC.

Tumor assessments were performed using dynamic computed tomography or magnetic resonance imaging every 3 months from baseline for 24 months, and then every 3–6 months in both groups. All scans were reviewed by 2 independent radiologists at each site with more than 5 years' experience, who were unaware of the group assignment. In cases of discordance, an additional third independent experienced radiologist reviewed images and consensus was achieved among the 3 radiologists. Adverse events (AEs), which were classified and graded according to the Common Terminology Criteria for Adverse Events, version 3.0, were assessed from the time the patient provided written informed consent until the end of the study or drop-out, and until at least 30 days after the last dose of immunotherapy. Multiple occurrences of specific events were counted once per patient; the event with the greatest severity was summarized. The data cut-off date was November 29, 2012.

Statistical Analysis

Sample size for the study was determined on the basis of the primary end point of RFS. Assuming a 1-sided type I error of 0.05, a power of 80%, and a randomization ratio for 1:1 between the 2 study groups, 57 recurrences or death events were required to expect a hazard ratio [HR] of 0.5, which was estimated from a 22% point increase (from 45% to 67%) in the 2-year RFS rate.[15] When the potential loss to follow-up rate was set at 20%, 160 patients were needed to record 57 recurrence events.

The interim analysis, which originally was planned for sample size re-estimation, was performed by an independent statistician using a cut-off date of November 30, 2009, by which time the prespecified 28 recurrence or death events (approximately 50% of projected events) had occurred. By using an interim hazard ratio, the adjusted hazard ratio was 0.58, indicating the need to increase the event threshold to 86. The loss to follow-up rate was adjusted to 4%. On the basis of these calculations, we re-estimated that we needed to enroll 230 patients.

The efficacy outcomes were assessed according to the intention-to-treat principle. Kaplan–Meier curves were generated for RFS, OS, and cancer-specific survival and the log-rank test was used for group comparisons. Unadjusted HRs were estimated using the Cox proportional hazards model. To compare the consistency of the effect of study treatment on the primary end point with immunotherapy and with no immunotherapy, we performed prespecified subgroup analyses as well as post hoc ones. A Cox proportional hazard

analysis was performed to assess the effect of baseline characteristics on each outcome of interest. AEs were compared between the 2 study groups using the chi-square test or the Fisher exact test. The log-rank test for the primary end point was 1-sided and all other statistical tests were 2-sided. Statistical significance was set at a P value of less than .05. The statistical analysis was performed by statisticians at the Department of Statistics of Korea University (Seoul, Korea) using SAS software version 9.2 (SAS Institute, Inc, Cary, NC); the R statistical programming environment, version 2.15.3 (http://www.r-project.org); and STATA software version 13.0 (StataCorp, College Station, TX).

Results

Patients

Between July 3, 2008, and November 29, 2012, there were 245 participants who were screened. A total of 230 eligible participants were assigned randomly to either the immunotherapy group (n = 115) or the control group (n = 115). Among these randomized patients, 226 (114 in the immunotherapy group and 112 in the control group) were included in the efficacy analysis: 4 patients were excluded from the efficacy analysis because 1 in the immunotherapy group and 3 in the control group were found to have violated the inclusion and exclusion criteria according to a decision from the steering committee. One patient in the immunotherapy group was lost to follow-up evaluation and 10 patients in the immunotherapy group discontinued intervention. Also, 15 patients in the control group were lost to follow-up (Supplementary Appendix). All 230 randomized patients were included in the safety population. At the time of the data cut-off date, the median follow-up duration was 40.0 months in the immunotherapy group and 36.5 months in the control group.

None of the differences in the baseline characteristics between the 2 study groups were statistically significant, except for platelet count (Table 1). Approximately 30% of patients underwent surgical resection. Chronic hepatitis B virus (HBV) infection was the predominant cause of liver disease and approximately two thirds of patients had liver cirrhosis. Time interval and modality of imaging studies were comparable between study groups (Supplementary Table 3). Patients in the immunotherapy group received the CIK cell agent containing an average of 6.4×10^9 cells per a treatment (Table 2).

Efficacy

Recurrence-free survival. The median RFS was 14.0 months longer in the immunotherapy group (44.0 mo) than in the control group (30.0 mo). The difference in RFS between the 2 groups was statistically significant ($P = .010$ by 1-sided log-rank test). Among the 226 patients in the efficacy population, a total of 101 patients experienced tumor recurrence or death by the time of the data cut-off date: 46 of the 114 patients (40%) in the immunotherapy group (45 recurrences and 1 death without recurrence) and 55 of the 112 patients (49%) in the control group (53 recurrences and 2 deaths without recurrence). The HR for tumor recurrence or death in the immunotherapy group vs the control group was 0.63 (95% confidence interval [CI], 0.43–0.94), representing a 37% relative risk reduction in the immunotherapy group (Figure 1*A* and Supplementary Table 4). The immunotherapy consistently reduced the risk of all 3 types of tumor recurrence: intrahepatic local recurrence (within 2 cm from resection or ablation margin), intrahepatic distant recurrence (beyond 2 cm from margin), and extrahepatic recurrence (Supplementary Figure 2).

On multivariate analysis using a stepwise selection method, adjuvant immunotherapy was proven to be a significant prognostic factor (adjusted HR, 0.66; 95% CI, 0.44–0.98; $P = .04$) after adjustment for age, serum level of α-fetoprotein, and treatment modality (Supplementary Table 5). Subgroup analyses according to prespecified and post hoc factors showed a benefit on RFS for adjuvant CIK immunotherapy over the control group in most of the subgroups analyzed (Figure 2).

Among 100 patients (45 in the immunotherapy group and 55 in the control group) who experienced tumor recurrence, patients underwent additional treatment for a median of 2 times (range, 0–17), with multidisciplinary modalities including surgical resection, RFA, PEI, transarterial chemoembolization, liver transplantation, external radiation therapy, proton therapy, sorafenib, and conventional cytotoxic chemotherapy (Supplementary Table 6).

Overall and cancer-specific survival. At the time of the data cut-off date, 15 deaths had occurred in the efficacy population: 3 patients in the immunotherapy group and 12 in the control group. In the immunotherapy group, patients died of recurrent HCC (2 patients) or new primary gastric cancer (1 patient). In the control group, patients died of recurrent HCC (9 patients) or unknown causes (3 patients). Both the median overall and cancer-specific survivals in both groups were not reached. OS was longer in the immunotherapy group than in the control group (HR, 0.21; 95% CI, 0.06–0.75; $P = .008$) (Figure 1*B*). Recurrence status significantly affected OS (relative risk of death, 5.22; 95% CI, 1.52–18.01; $P = .003$ by z-test). In addition, cancer-specific survival was longer in the immunotherapy group (HR, 0.19; 95% CI, 0.04–0.87; $P = .02$) (Figure 1*C* and Supplementary Table 4).

Safety

AEs were reported for 118 patients (51%) in the safety population and were mild to moderate (grade 1 or 2) for 109 patients (47%). Overall, AEs occurred more frequently in the immunotherapy group (62%) than in the control group (41%) ($P = .002$), but the frequency of grade 3 or 4 AEs was comparable between 2 study groups ($P = .18$). Chills, pyrexia, and productive cough were reported more frequently in the immunotherapy group (Table 3). The frequency of serious AEs between 2 groups were comparable (7.8% in the immunotherapy group vs 3.5% in the control group; $P = .15$).

The CIK cell agent–related adverse drug reactions including pyrexia, chills, myalgia, and fatigue were reported

Table 1. Baseline Demographics and Disease Characteristics

Variable	Immunotherapy (n = 114)	Control group (n = 112)	*P* value
Sex, N (%)			NS[a]
Male	95 (83.3)	91 (81.3)	
Female	19 (16.7)	21 (18.8)	
Age, mean (SE), *y*	55.4 (8.2)	56.4 (10.6)	NS[b]
Treatment modality, N (%)			NS[c]
PEI	13 (11.4)	4 (3.6)	
RFA	69 (60.5)	70 (62.5)	
Surgical resection	32 (28.1)	38[d] (33.9)	
HCC stage, N (%)[e]			NS[a]
Stage I	98 (86.0)	94 (83.9)	
Stage II	16 (14.0)	18 (16.1)	
Number of HCC, N (%)			NS[c]
≥3	2 (1.8)	2 (1.8)	
<3	112 (98.2)	110 (98.2)	
Size of HCC, *cm*			.02[f]
Median	1.8	2.3	
IQR (Q3–Q1)	1.4–2.3	1.5–3.1	
ECOG performance status, N (%)[g]			NS[a]
0	81 (71.1)	81 (72.3)	
1	33 (28.9)	31 (27.7)	
Cause of liver disease, N (%)			NS[c]
HBV infection only	96 (84.2)	90 (80.4)	
HCV infection only	9 (7.9)	10 (8.9)	
HBV and HCV co-infection	2 (1.8)	2 (1.8)	
Others	7 (6.1)	10 (8.9)	
Cirrhosis, N (%)[h]	76 (66.7)	70 (62.5)	NS[a]
Biochemical analysis			
α-fetoprotein level, *ng/mL*			NS[f]
Median	5.2	5.4	
IQR (Q1–Q3)	3.1–9.9	3.0–13.0	
PIVKA-II, *mAU/mL*			NS[f]
Median	19.0	18.0	
IQR (Q3–Q1)	14.0–24.8	14.0–24.0	
Aspartate aminotransferase level, *IU/L*			NS[f]
Median	33.0	34.0	
IQR (Q1–Q3)	27.0–43.5	26.8–44.0	
Alanine aminotransferase level, *IU/L*			NS[f]
Median	33.0	33.0	
IQR (Q1–Q3)	25.0–45.8	23.0–47.5	
Alkaline phosphatase level, *IU/L*			NS[f]
Median	82.5	82.0	
IQR (Q1–Q3)	70.0–101.5	65.0–100.0	
Albumin level, *g/dL*			NS[f]
Median	4.1	4.1	
IQR (Q1–Q3)	3.9–4.3	3.9–4.3	
Total bilirubin level, *mg/dL*			NS[f]
Median	0.8	0.8	
IQR (Q1–Q3)	0.6–1.0	0.6–1.0	
Prothrombin time, *s*			NS[f]
Median	13.7	13.9	
IQR (Q1–Q3)	13.1–14.7	13.2–14.4	
Creatinine level, *mg/dL*			NS[f]
Median	0.9	0.9	
IQR (Q1–Q3)	0.8–1.0	0.7–1.0	
Platelet, $\times 10^3/mm^3$			.01[f]
Median	116.5	141.0	
IQR (Q1–Q3)	92.3–158.0	117.5–166.3	

NOTE. Data are expressed as n (%), mean (SE), or median with interquartile range.
ECOG, Eastern Cooperative Oncology Group; HCV, hepatitis C virus; IQR, interquartile range; PIVKA-II, protein induced by vitamin K absence-II.
[a]Chi-square test.
[b]By 2-sample t test.
[c]Fisher exact test.
[d]Two of them underwent intraoperative RFA in addition to surgical resection.
[e]The HCC staging was performed according to the American Joint Committee on Cancer staging system (6th ed).[21]
[f]Wilcoxon rank-sum test.
[g]The ECOG performance status assesses the daily living abilities of the patient, on a scale ranging from 0 (fully active) to 5 (dead).
[h]Cirrhosis was diagnosed by the presence of histologic and radiologic evidence.

in 19 patients (17%), however, they did not delay or stop the treatment. One patient in the immunotherapy group dropped out because of serious AEs (new primary gastric cancer), which, however, was not assessed as an adverse drug reaction.

Discussion

In this trial, patients who received an adjuvant immunotherapy using the CIK cell agent after curative treatment for HCC had a 14-month median RFS benefit, as compared with those who received no adjuvant immunotherapy. At the final analysis, patients in the immunotherapy group had a median RFS of 44.0 months, as compared with 30.0 months in the control group. The effect of the CIK cell agent on RFS remained significant after adjustment for baseline prognostic factors of recurrence.

This trial showed that adjuvant immunotherapy improved overall and cancer-specific survival in HCC patients. The magnitude of absolute survival gain in the immunotherapy group was modest, but the relative risk reduction was significant (approximately four-fifths relative risk reduction of both overall and cancer-specific mortalities in the immunotherapy group). In contrast, previous studies using uncommercialized CIK cells showed

Table 2. Summary of Injected CIK Cell Agents: Safety Population

	Immunotherapy (n = 115)
Total cell count, $\times 10^9$	
Mean ± SD	6.4 ± 2.1
Range	1.4–20.0
Cell viability, %	
Mean ± SD	97.4 ± 2.2
Range	90–100
$CD3^+$ cell, %	
Mean ± SD	98.3 ± 2.5
Range	80.3–100
$CD8^+$ cell, %	
Mean ± SD	83.8 ± 7.9
Range	60.1–98.4
$CD56^+$ cell, %	
Mean ± SD	27.1 ± 9.9
Range	10–76.9
$CD14^+$ cell, %	
Mean ± SD	0.1 ± 0.1
Range	0.0–0.7
$CD20^+$ cell, %	
Mean ± SD	0.1 ± 0.1
Range	0.0–0.9
Injection times	
0	5 (4.4%)[a]
1–3	4 (3.5%)
4–7	2 (1.7%)
8–11	14 (12.2%)
12–15	7 (6.1%)
16	83 (72.2%)
Total	1569

NOTE. Data are expressed as mean ± SD, range, or n (%).
[a]Three patients withdrew informed consent, 1 patient failed blood collection, and 1 patient had a protocol violation.

significant benefits in preventing recurrence, but no significant survival gains.[15–17] The intensified schedule of CIK cell agent administration and favorable tumor characteristics in our study may account for the improved OS in contrast to previous trials. CIK cells were infused more times (16 times) in our study than in preceding studies (3–10 times). In our study, only patients with American Joint Committee on Cancer clinical stage I or II HCC were included, whereas preceding studies included patients with more advanced stage tumor (ie, high proportion of stage III or IV tumors, 46%[15]; tumors with vascular invasion, 46%[17]; or large HCCs of > 5 cm, >60%[16]). Patients with greater tumor burden in those preceding studies might have had increased numbers of immune-suppressor cells that can attenuate the effect of adjuvant immunotherapy,[24,25] and thus might have impeded the survival benefit. In addition, our study used commercialized CIK cell agents manufactured in a Good Manufacturing Practice-certified central facility following standard operating procedures under strict quality control and assurance, whereas the CIK cell preparation was performed by their own cultivating methods in the previous studies.

HCC development and progression is well known to be related to chronic inflammation.[26] After tumor cells are

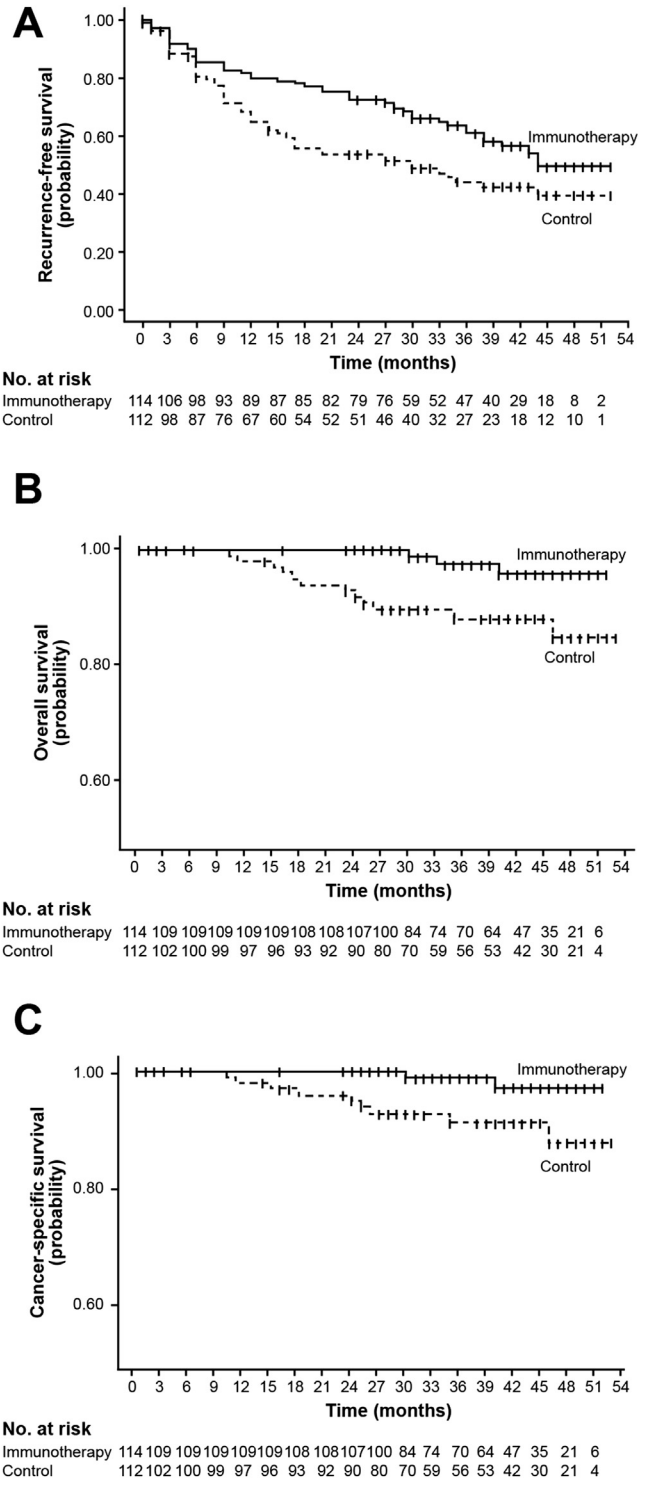

Figure 1. Kaplan–Meier estimates of RFS, OS, and cancer-specific survival. (*A*) RFS was computed for all patients included in the efficacy population. Patients who had not progressed or died were censored on the data cut-off date. (*B*) OS was computed for all patients included in the efficacy population. Patients who had not died were censored on the data cut-off date. (*C*) Cancer-specific survival was computed for all patients included in the efficacy population. Patients who had not died of hepatocellular carcinoma were censored on the date of death owing to other causes or the data cut-off date.

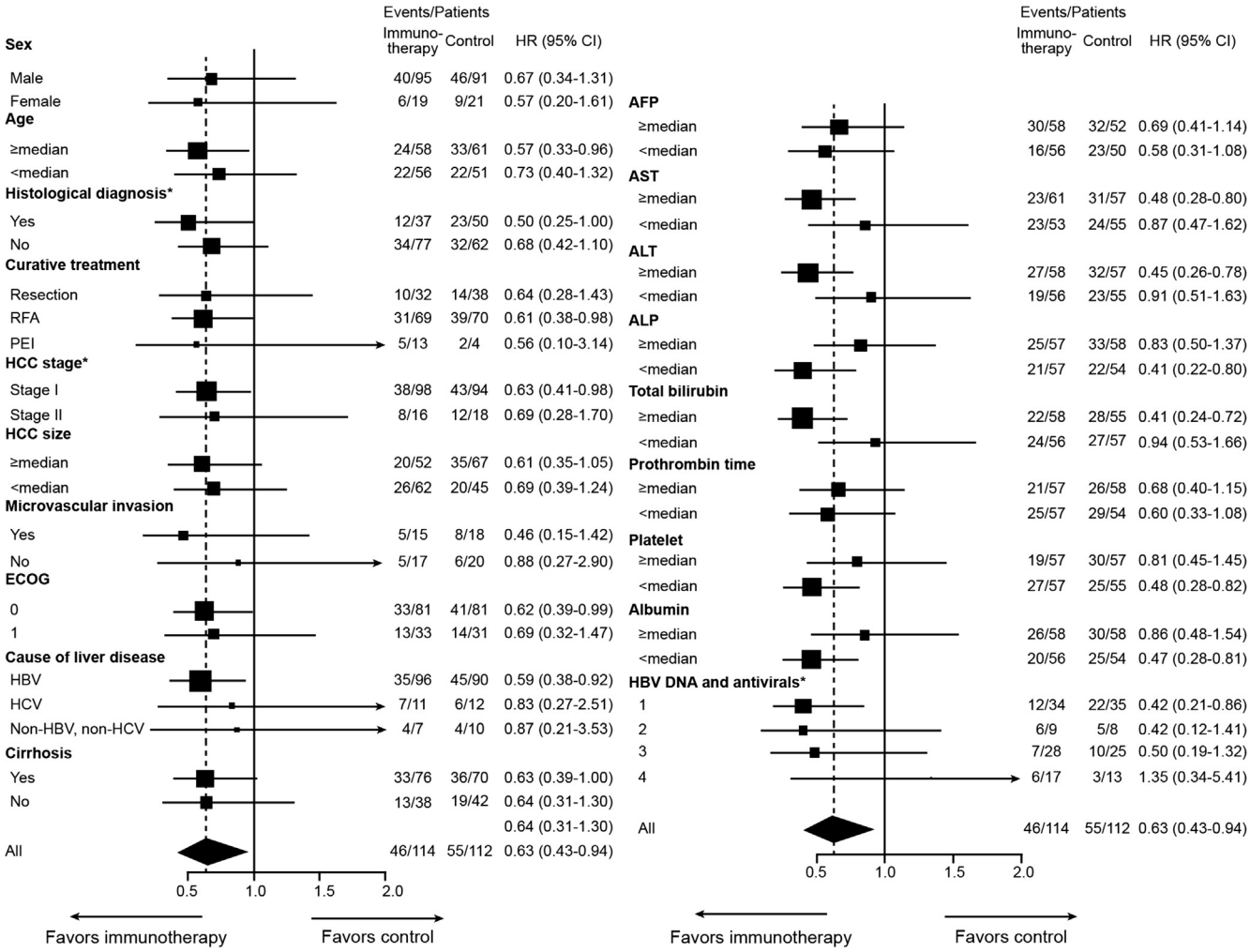

Figure 2. Recurrence-free survival in selected subsets. The graph show the estimates of the HR for every subgroup as a square (whose size is proportional to the amount of information) and the horizontal lines depict the 95% CIs, which were calculated by means of a Cox proportional hazards model. The *diamond* indicates the HRs with 95% CIs for all patients enrolled. The *vertical solid line* at the HR of unity corresponds to the line of no effect. HR values of less than unity correspond to a reduction in the risk of recurrence or death with immunotherapy. Patients co-infected with both HBV and HCV were included in the HCV subset. HBV DNA and antiviral agent groups 1, 2, 3, and 4 indicate patients with serum HBV-DNA levels $\geq$ 2000 IU/mL who underwent antiviral treatment; those with serum HBV-DNA levels $\geq$ 2000 IU/mL who underwent no antiviral treatment; those with HBV-DNA levels $<$ 2000 IU/mL who underwent antiviral treatment; and those with HBV-DNA levels $<$ 2000 IU/mL who underwent no antiviral treatment, respectively. *Characteristics were post hoc subgroups. AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ECOG, Eastern Cooperative Oncology Group; HCV, hepatitis C virus.

established, mutual interactions between tumor cells and the immune cells present during chronic inflammation may create conditions more favorable for tumor cell survival.[27] Immune-suppressor cells (eg, tumor-associated macrophages, regulatory T cells, or myeloid-derived suppressive cells) facilitate tumor immune evasion.[28] Effector cells (eg, dendritic cells, cytotoxic T cells, and NK cells) decrease and their effectiveness is impaired in the tumor microenvironment.[29] In addition, because growing tumors acquire mutations to evade the immune system[30] and antigen-presenting cells and $CD8^+$ T cells are functionally impaired, major histocompatibility complex (MHC)-restricted cytotoxic immunity is incapacitated.[31] To overcome the aforementioned limitations of the cytotoxic immune response against HCC, potentially effective strategies should include enhancing MHC-unrestricted direct cytotoxic effector cells; thus, we selected $CD3^+/CD56^+$ CIK cells as a potential answer. Within a certain period of in vitro incubation with IL2 and anti-CD3 antibody, the precursors ($CD3^+$ T cells) can acquire an MHC-unrestricted, cell-mediated cytotoxicity in addition to T-cell receptor–mediated cytotoxicity after gaining CD56.[32–34] Approximately 27% of infused lymphocytes had CD56 in our study. Compared with $CD3^+/CD56^-$ T cells, $CD3^+/CD56^+$ CIK cells have a higher proportion of $CD8^+$ cells and a higher granzyme content.[35] Consequently, $CD3^+/CD56^+$ CIK cells exert

Table 3. Adverse Events: Safety Population

	Immunotherapy (n = 115)				Control (n = 115)			
	All AE		Related AE		All AE		*P* value	
Adverse event	Any grade	Grade 3 or 4	Any grade	Grade 3 or 4	Any grade	Grade 3 or 4	Any grade	Grade 3 or 4
Overall incidence	71 (62%)	7 (6%)	40 (35%)	0	47 (41%)	4 (4%)	.002[a]	.354[a]
Vomiting	3 (3%)	0	1 (1%)	0	3 (3%)	1 (1%)	1.00[b]	1.00[b]
Chills	10 (9%)	0	9 (8%)	0	0	0	.001[a]	NA[b]
Fatigue	11 (10%)	0	3 (3%)	0	3 (3%)	0	.03[a]	NA[b]
Pyrexia	13 (11%)	0	10 (9%)	0	0	0	<.001[a]	NA[b]
URI	7 (6%)	0	0	0	3 (3%)	0	.20[a]	NA[b]
Headache	3 (3%)	0	2 (2%)	0	1 (1%)	1 (1%)	.62[b]	1.00[b]
Productive cough	6 (5%)	0	0	0	0	0	.03[b]	NA[b]

NOTE. Listed are adverse events, as defined by the National Cancer Institute Common Terminology Criteria (version 3.0), that were considered drug-related or that occurred in at least 3 patients in either study group regardless of relationship to drug. Data are expressed as N (%).
AE, adverse event; URI, upper respiratory tract infection.
[a]Chi-square test.
[b]Fisher exact test.

more potent antitumor toxicity than $CD3^+/CD56^-$ T cells in in vitro studies.[35,36] $CD3^+/CD56^+$ CIK cells kill tumor cells with granzyme and perforin-mediated tumor cell lysis after tumor recognition.[37–39]

As shown in Figure 1*A*, our study as well as the previous 3 trials consistently showed that CIK cell treatment improves RFS by reducing the risk of early recurrence (within the first 2 years), but fails to affect late recurrence (beyond 2 years).[15–17] Because early recurrence of HCC is related closely to metastasis of remnant neoplastic cells rather than de novo hepatocarcinogenesis,[40] clearing residual HCC cells using CIK cells might explain the reduced early recurrence, which consequently improved RFS. The CIK cell immunotherapy reduced all types of tumor recurrence: local intrahepatic recurrence, distant intrahepatic recurrence, and extrahepatic recurrence. In addition to the direct tumor-killing effect of the CIK cell agent, there also could be an indirect mechanism of reducing tumor recurrence by controlling the replication of HBV, which was the predominant cause of HCC in this study. It has been reported that autologous CIK cells could suppress HBV replication,[41] which could reduce the risk of HCC recurrence in HBV-related HCC patients.[42,43] However, the effect of CIK cells on the hepatitis C virus has not been evaluated fully and further studies are required.

Further studies comparing the pretreatment factors and post-treatment immunologic responses generated by the CIK cell agent between the responders and the non-responders also are warranted because the results might enable the stratification of patients who might derive more benefit from CIK cell immunotherapy, and also might enhance the efficacy of CIK cell therapy. Recent studies have suggested several potential biomarkers including the post-treatment CD4/CD8 ratio, the percentages of T cells and NK cells, and B7 family molecules.[44–46]

Although this trial was a randomized trial, there were several baseline characteristics that were imbalanced between study groups: tumor size, platelet count, and prior curative treatment modality. The immunotherapy group had significantly smaller tumors, which might favor the immunotherapy group. In contrast, a significantly lower platelet count and a marginally higher proportion of ablative therapy (RFA and PEI) in the immunotherapy group might have had a negative impact on the effectiveness of immunotherapy.[47,48] In preplanned multivariate analysis and subgroup analysis, those imbalanced baseline characteristics were proven to have no significant impact on the effectiveness of CIK cell immunotherapy. In our study, patients were stratified solely by treatment center. In a future study of adjuvant therapy, to avoid both overstratification and imbalance among important prognostic factors, it would be better to stratify patients according to several key prognostic factors such as tumor size and treatment modality.[49]

The overall differences in serum aminotransferase levels between the 2 study groups were not significantly different (Supplementary Table 7), which indicates that repeated transfer of CIK cells did not cause significant hepatocellular injury. Although overall AEs were more frequent in the immunotherapy group, they were mainly grades 1 or 2 in severity and the frequency of serious AEs was comparable between both groups. Most adverse drug reactions were not linked to stopping or delaying adjuvant treatment. These results collectively suggested that treatment with the CIK cell agent was safe and well tolerated.

In conclusion, this study showed that adjuvant CIK cell immunotherapy prolongs RFS and OS in patients who have undergone curative treatment for HCC. The immunotherapy was associated with a higher frequency of AEs, which were mainly mild to moderate.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.02.055.

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Conflicts of interest
These authors disclose the following: Joon Hyeok Lee has received grants from Green Cross Cell Corp, Bukwang Pharmaceuticals, Pfizer, JW Creagene, and GlaxoSmithKline, has received lecture fees from Bayer Pharmaceuticals, Gilead Science, Bukwang Pharmaceuticals, and GlaxoSmithKline, and serves as an advisory board member of Gilead Science and Bristol-Myers Squibb; Jung-Hwan Yoon has received a research grant from Bayer HealthCare Pharmaceuticals; Young-Suk Lim has received research grants from Bayer HealthCare Pharmaceuticals and Gilead Science; Jong Eun Yeon has received research grants from Yuhan Pharmaceuticals and Jeil Pharmaceuticals, and serves as an advisory board member of Bayer Korea; Yoon Jun Kim has received research grants from Bristol-Myers Squibb, Roche, JW Creagene, Bukwang Pharmaceuticals, Handok Pharmaceuticals, Hanmi Pharmaceuticals, Yuhan Pharmaceuticals, and Pharmaking, and has received lecture fees from Bayer HealthCare Pharmaceuticals, Gilead Science, MSD Korea, Yuhan Pharmaceuticals, Samil Pharmaceuticals, CJ Pharmaceuticals, Bukwang Pharmaceuticals, and Handok Pharmaceuticals; Kang Mo Kim has received research grants from AstraZeneca, Yuhan Pharmaceuticals, and Il-Dong Pharmaceuticals, has received lecture fees from Bayer HealthCare Pharmaceuticals, MSD, and GlaxoSmithKline, and has received a consulting fee from Bayer HealthCare Pharmaceuticals; Geum-Youn Gwak has received lecture fees from Bristol-Myers Squibb, JW Creagene, Handok Pharmaceuticals, and MSD Korea; and Su Jong Yu has received a lecture fee from Bayer HealthCare Pharmaceuticals. The remaining authors disclose no conflicts.

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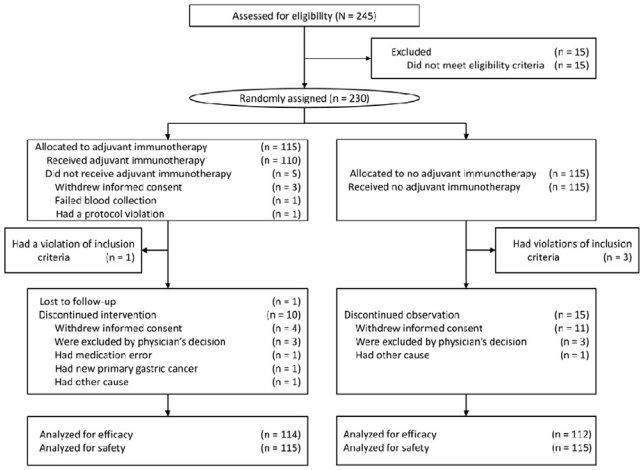
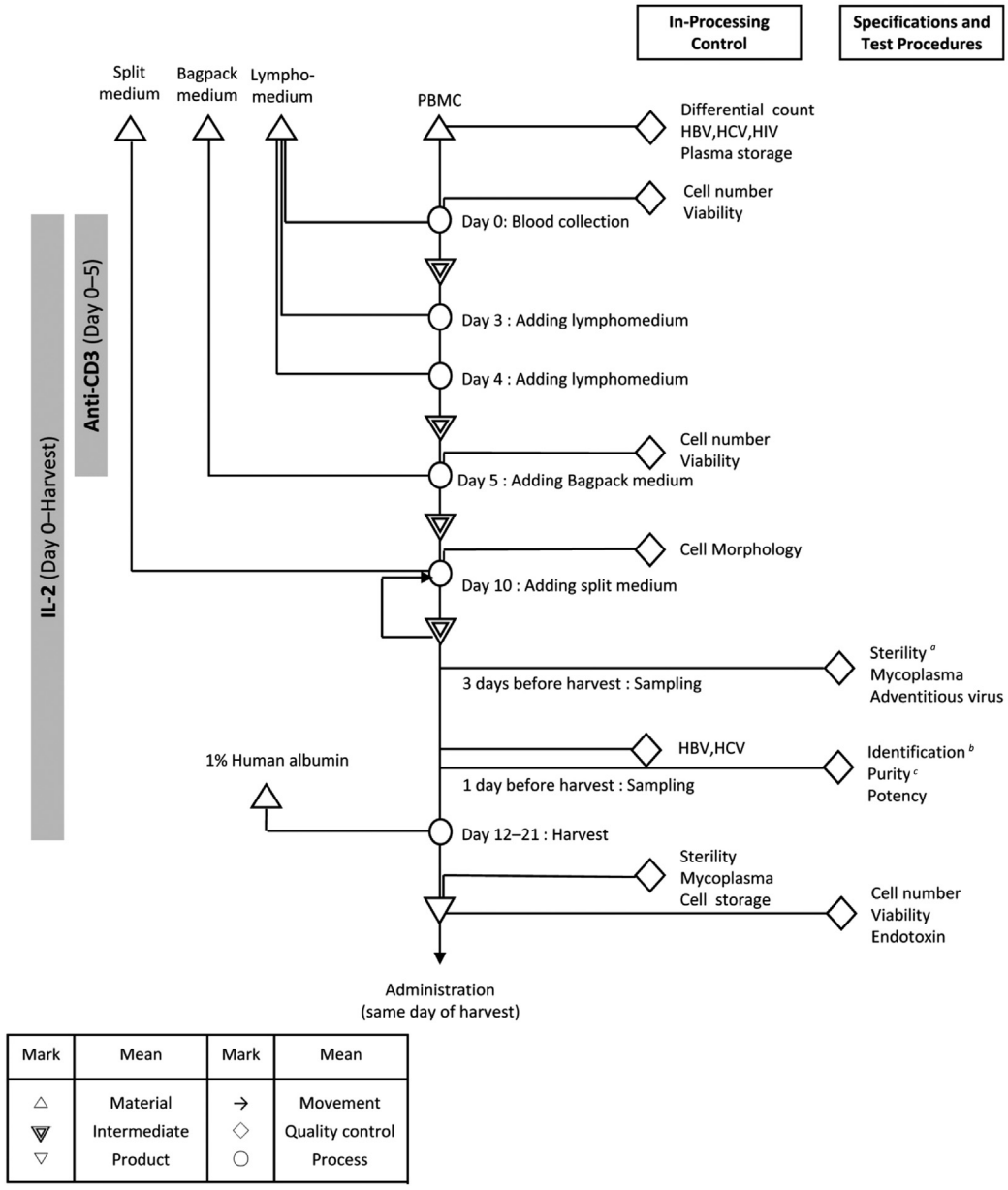
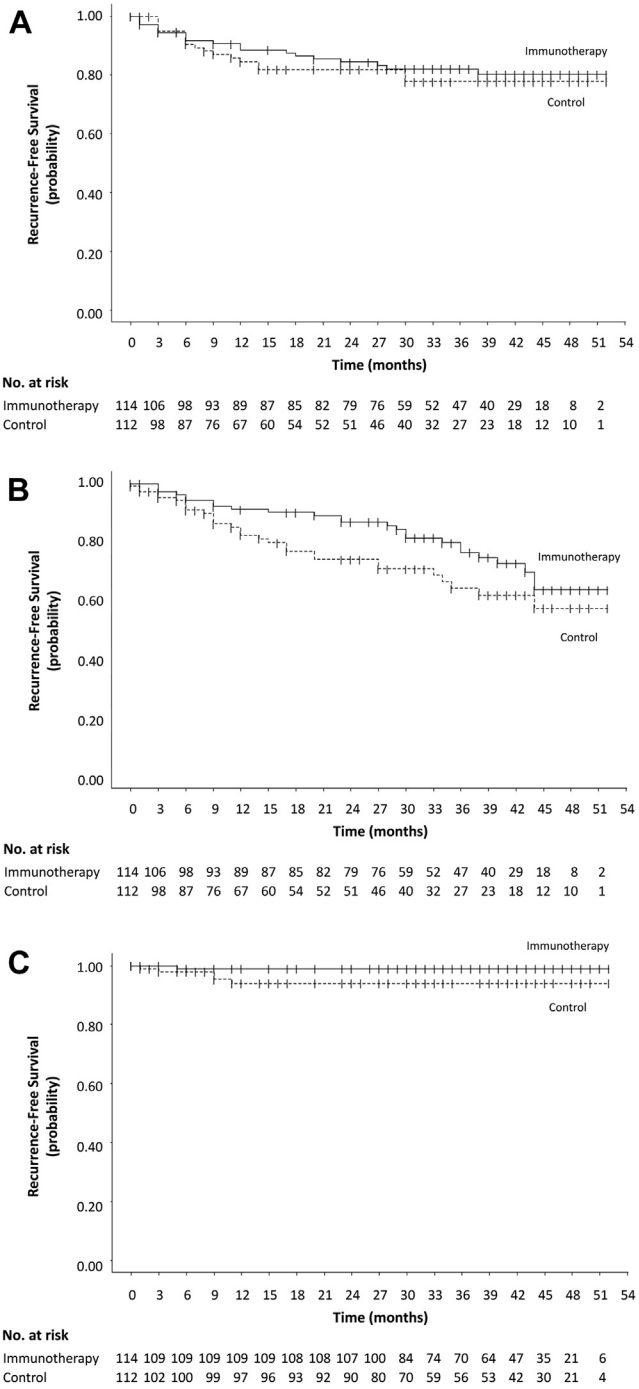


Supplementary Appendix. CONSORT diagram. Of the enrolled patients, 15 did not meet inclusion criteria and thus were excluded from random assignment. In addition, 4 more patients were excluded from the efficacy analysis because they were found to violate inclusion criteria after randomization according to a decision from the steering committee.



Supplementary Figure 1. Manufacturing process of CIK cell agent. After collection of peripheral blood from the respective patients (day 0), mononuclear cells were separated and cultured for 12–21 days with interleukin 2 (from day 0 to the date of harvest) and anti-CD3 monoclonal antibody (from day 0 to day 5). Tests for sterility and mycoplasma were performed twice at 3 days before harvest and at the date of harvest. Tests for identification, purity, and potency were performed 1 day before harvest. At the date of harvest, CIK cell agents were administered to patients. HCV, hepatitis C virus; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell. [a]Sterility test included aerobic and anaerobic bacteria and fungi. [b]Identification included proportions of $CD3^+$, $CD8^+$, and $CD56^+$ cells. [c]Purity test included proportions of $CD14^+$ and $CD20$ $CD3^+$cells.



Supplementary Figure 2. Kaplan–Meier estimates of RFS according to the respective recurrence site. (*A*) Intrahepatic local recurrence (within 2 cm from surgical or ablation margin: HR, 0.85; 95% CI, 0.44–1.62; $P = .30$ by 1-sided log-rank test), (*B*) intrahepatic distant recurrence (beyond 2 cm from margin: HR, 0.63; 95% CI, 0.37–1.07; $P = .04$ by 1-side log-rank test), and (*C*) extrahepatic recurrence (HR, 0.17; 95% CI, 0.02–0.15; $P = .03$ by 1-side log-rank test). RFS was computed on all patients included in the efficacy population. Patients who had not progressed or died were censored on data cut-off date.

Supplementary Table 1. The Definition of Hepatocellular Carcinoma of Pretreatment Clinical Stage I or II According to the American Joint Committee on Cancer Staging System Sixth Edition

Stage	
I	A solitary tumor without vascular invasion, lymph node metastasis, and distant metastasis
II	A solitary tumor with vascular invasion, but without lymph node metastasis, and distant metastasis; or multiple tumors, none more than 5 cm, without vascular invasion, lymph node metastasis, and distant metastasis

NOTE. Clinical stage was based primarily on the radiologic evaluation before treatment.

Supplementary Table 2. The Criteria of "Appropriate Manufacture of Biologic Agents" Following the Guidance from US Food and Drug Administration, Center for Biologics Evaluation and Research

Sterility test	No growth of aerobes, anaerobes, and fungi
Mycoplasma test	Negative
Endotoxin test	Negative
Viability and content	Proportion of viable cells, $\geq$ 90% Total cell counts, 1.0×10^9–2.0×10^{10} cells

NOTE. (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074131.htm).

Supplementary Table 3. Summary of Imaging Studies

	Immunotherapy (n = 114)	Control group (n = 112)	*P* value
Interval between each imaging study, *days* (per participants)			
Before 96 weeks, median (range)	78 (41–90)	77 (41–84)	.10[a]
After 96 weeks, median (range)	114 (74–182)	113 (63–168)	.32[a]
Number of overall imaging studies, N (per participants)			<.001[b]
Mean ± SD	9.0 ± 4.6	7.1 ± 4.8	
Number of each imaging modalities, N (%) (overall participants)			.68[c]
CT	1106 (98.2%)	887 (98.5%)	
MRI	20 (1.8%)	13 (1.5%)	

CT, computed tomography; MRI, magnetic resonance imaging.
[a]Wilcoxon rank-sum test.
[b]Two-sample *t* test.
[c]Chi-square test.

Supplementary Table 4. Summary of Efficacy Measures: Efficacy Population

Outcome	Immunotherapy (n = 114)	Control (n = 112)	Hazard ratio (95% CI)	*P* value
Recurrence-free survival rate				
12 months	79.9%	65.1%		
24 months	72.5%	53.8%		
36 months	60.9%	44.3%		
48 months	49.6%	39.6%		
Recurrence-free survival, *mo*			0.63 (0.43–0.94)	.010
Median	44.0	30.0		
Overall survival rate				
12 months	100.0%	98.0%		
24 months	100.0%	91.8%		
36 months	97.5%	88.1%		
48 months	95.9%	84.8%		
Overall survival, *mo*			0.21 (0.06–0.75)	.008
Median	NA	NA		
Cancer-specific survival rate				
12 months	100.0%	98.0%		
24 months	100.0%	94.9%		
36 months	98.8%	91.0%		
48 months	97.2%	87.5%		
Cancer-specific survival, *mo*			0.19 (0.04–0.87)	.020
Median	NA	NA		

NOTE. Data are expressed as a percentage, hazard ratio with 95% CI, or median.

Supplementary Table 5. Univariate and Multivariate Analyses of Factors Associated With Recurrence-Free Survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age, ≥60 vs <60 y	1.58 (1.06–2.34)	.02	1.52 (1.01–2.28)	.04
Sex, male vs female	1.17 (0.68–2.03)	.84		
Etiology of liver disease, HBV or HCV vs NBNC	0.82 (0.40–1.69)	.59		
Cirrhosis, yes vs no	1.26 (0.82–1.92)	.28		
AFP level, ≥20 vs <20 ng/mL	1.74 (1.04–2.89)	.03	2.12 (1.24–3.60)	.006
ECOG performance status, 0 vs 1	1.40 (0.90-2.18)	.71		
Histologic confirmation, yes vs no	0.85 (0.57–1.28)	.43		
HCC maximal diameter, ≥2 vs <2 cm	1.20 (0.81–1.78)	.34		
Treatment modality, RFA or PEI vs surgery	1.50 (0.94–2.37)	.054	1.75 (1.08–2.83)	.02
Platelet count, ≥140 vs <140 × 10^3/mm^3	0.93 (0.62–1.38)	.70		
AST level, ≥40 vs <40 IU/L	1.43 (0.96–2.12)	.07		
ALT level, ≥40 vs <40 IU/L	1.01 (0.67–1.52)	.96		
ALP level, ≥115 vs <115 IU/L	1.11 (0.65–1.90)	.69		
PIVKA-II level, ≥40 vs <40 AU/mL	0.71 (0.26–1.95)	.50		
Albumin level, ≥3.5 vs <3.5 g/dL	1.18 (0.48–2.92)	.71		
Bilirubin level, ≥1.2 vs <1.2 mg/dL	1.11 (0.66–1.87)	.69		
Prothrombin time, ≥13 vs <13 s	1.01 (0.60–1.70)	.98		
Creatinine level, ≥1.2 vs <1.2 mg/dL	1.15 (0.53–2.48)	.72		
Treatment group, immunotherapy vs control group	0.63 (0.43–0.94)	.01[a]	0.66 (0.44–0.98)	.04

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ECOG, Eastern Cooperative Oncology Group; HCV, hepatitis C virus; NBNC, non-HBV and non–hepatitis C virus; PIVKA-II, protein induced by vitamin K absence-II.
[a]By a 1-sided test. Otherwise, a 2-sided test was used.

Supplementary Table 6. Postrecurrence Treatment Modalities in the Immunotherapy Group and the Control Group

Treatment modalities	Immunotherapy (n = 45)	Control (n = 55)
Transarterial chemoembolization	98	126
Radiofrequency ablation	25	29
Percutaneous ethanol injection	21	17
Surgical resection	4	6
Liver transplantation	2	2
Sorafenib	3	4
Conventional chemotherapy	2	1
Radiation therapy	4	4
Proton therapy	0	1
Total	159	190

Supplementary Table 7. Changes in Serum Levels of α-Fetoprotein, Aspartate Aminotransferase, and Alanine Aminotransferase: Efficacy Population

Variable	Immunotherapy (n = 114)	Control (n = 112)	*P* value[a]
α-fetoprotein level, *ng/mL*			
Baseline	11.3 (18.6)	9.2 (13.6)	
End of study	14.5 (38.9)	53.1 (439.4)	
Change from baseline	3.6 (40.4)	49.3 (463.3)	.58
P value[b]	.12	.48	
Aspartate aminotransferase level, *IU/L*			
Baseline	41.9 (30.9)	39.0 (18.1)	
End of study	38.0 (23.6)	37.5 (20.2)	
Change from baseline	-3.8 (32.2)	-1.3 (18.9)	.83
P value[b]	.07	.10	
Alanine aminotransferase level, *IU/L*			
Baseline	41.5 (36.4)	40.2 (22.1)	
End of study	35.1 (23.6)	38.8 (29.8)	
Change from baseline	-6.4 (35.7)	-0.6 (25.8)	.52
P value[b]	.01	.12	

NOTE. Data are expressed as mean (SD).
[a]Wilcoxon rank-sum test comparing changes in the indicated parameters of 2 groups from baseline.
[b]Wilcoxon signed-rank test comparing changes in the indicated parameters within each group from baseline.